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CA 2552729 C 2013/07/16

(11)(21) **2 552 729**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2004/12/22
(87) Date publication PCT/PCT Publication Date: 2005/07/21
(45) Date de délivrance/Issue Date: 2013/07/16
(85) Entrée phase nationale/National Entry: 2006/07/06
(86) N° demande PCT/PCT Application No.: DK 2004/000896
(87) N° publication PCT/PCT Publication No.: 2005/066338
(30) Priorité/Priority: 2004/01/08 (DK PA 2004 00021)

(51) Cl.Int./Int.Cl. *C12N 9/30* (2006.01),
C12N 15/56 (2006.01)

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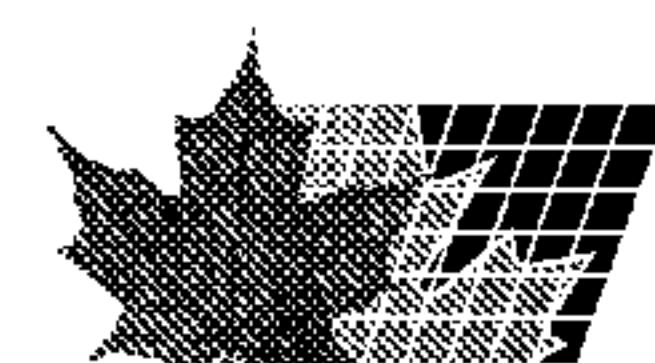
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(54) Titre : AMYLASE
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(57) **Abrégé/Abstract:**

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
21 July 2005 (21.07.2005)

PCT

(10) International Publication Number
WO 2005/066338 A1

- (51) International Patent Classification⁷: C12N 9/30, 15/56 (74) Common Representative: NOVOZYMES A/S; Krogshøjvej 36, DK-2880 Bagsværd (DK).
- (21) International Application Number: PCT/DK2004/000896 (81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 22 December 2004 (22.12.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: PA 2004 00021 8 January 2004 (08.01.2004) DK (84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
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- Published:
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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AMYLASE

FIELD OF THE INVENTION

The present invention relates to amylases and their addition to dough to prevent staling of dough-based products.

5 BACKGROUND OF THE INVENTION

Endo-amylases are often added to dough to make the dough more soft and to improve the moistness, often in combination with an exo-amylase, e.g. a maltogenic alpha-amylase. However, the addition of endo-amylases of bacterial origin, e.g. *B. amyloliquefaciens* amylase easily gives a risk of overdosing, giving loss of elasticity and a too gummy crumb.

10 Fungal amylases are often added to dough, but they only provide little effect on the softness

A fungal amylase (Taka amylase) from *Aspergillus oryzae* is described in Pdb file 6taa.

SUMMARY OF THE INVENTION

The inventors have identified an amylase in fungal strains of *Valsaria* and found that
15 the amylase can increase the shelf life of baked products. Particularly, the novel amylase in combination with an exo-amylase further improves the anti-staling effect of the exo-amylase. The improved anti-staling may be an improved the softness of bread crumb without a detrimental effect on crumb elasticity or even with an improved elasticity.

The amino acid sequence of the novel amylase was found to include a catalytic do-
20 main and a carbohydrate-binding domain (CBM), each of which can be used separately. Accordingly, the invention provides a polypeptide with a sequence including a catalytic core and a polypeptide with a sequence including a carbohydrate-binding domain (CBM).

The novel amylase is thermostable, and the inventors found that a combination of two amylases can be used for anti-staling, where one is a thermostable amylase (particularly a
25 fungal amylase) which includes both a catalytic core and a CBM, and the other is an exo-amylase.

The invention also provides a polypeptide having an amino acid sequence which can be obtained from the mature polypeptide (particularly the catalytic core of SEQ ID NO: 2 or 19 by substitution, deletion, and/or insertion of one or more amino acids and a polynucleotide hav-
30 ing a sequence that can be derived from SEQ ID NO: 1 or 18 by substitution, deletion, and/or insertion of one or more nucleotides.

The invention also provides a polynucleotide encoding the amylase, an expression vector comprising the polynucleotide, a transformed host cell comprising the vector, as well as a method of producing the amylase by cultivating the transformant. The invention further pro-

vides a dough composition comprising the amylase, a method of preparing a dough-based product by leavening and heating the dough, e.g. by baking.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

5 A source organism of the amylase of the invention is a fungal strain isolated from soil samples collected from Hainan Province, China, in 2002. The strain was at first classified as *Chaetomium* sp. and was later re-classified as *Valsaria rubricosa* belonging to Diaporthales, Ascomycetes, Ascomycota. It was found to harbor an amylase gene shown in SEQ NO: 1. The inventors have cloned the gene into a strain of *E. coli* and deposited it under the terms of the
10 Budapest Treaty on 16 December 2003 as DSM 16113 with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE.

The inventors also found an amylase gene in *Valsaria rubricosa* CBS 848.96. This strain is available on commercial terms from Centraalbureau voor Schimmelcultures, Uppsala-
15 laan 8, P.O. Box 85167, 3584 CT, The Netherlands. *Valsaria rubricosa* CBS 848.96 was found to harbor an amylase gene shown in SEQ NO: 18

Polypeptide with amylase activity

The polypeptide with amylase activity has an amino acid sequence which includes a catalytic core which may be the catalytic core sequence encoded by a DNA sequence in a
20 plasmid present in *E. coli* DSM 16113, the sequence shown in positions 1-439 of SEQ ID NO 2, or a sequence having at least 70 % identity to said sequences. Also, the catalytic core may be a sequence encoded by a nucleic acid sequence which hybridizes at 55 °C with the complementary strand of nucleotides 146-1462 of SEQ ID NO: 1.

Optionally, the amino acid sequence may further comprise both the catalytic domain
25 and a carbohydrate-binding module (CBM), or it may include the catalytic domain without a CBM. The CBM may be that present in the donor strain or an analogue thereof, or an extrinsic CBM may be substituted, or one or more additional CBMs may be inserted.

The polypeptide may have the sequence shown as SEQ ID NO: 19.

Carbohydrate-binding domain (CBM)

30 The carbohydrate-binding module (CBM) or carbohydrate-binding domain (CBD) is a polypeptide which binds preferentially to a poly- or oligosaccharide (carbohydrate), particularly in water-insoluble (e.g. crystalline) form. It may particularly be a starch-binding modules (SBM) or starch-binding domain (SBD). The one or more CBMs may optionally further comprise one

or more polypeptide amino acid sequence regions linking the CBM(s) with the catalytic module(s), a region of the latter type usually being denoted a "linker".

The invention provides a polypeptide with an amino acid sequence including a CBM with at least 70 % identity to amino acids 440-566 of SEQ ID NO: 2.

5 Heterologous carbohydrate-binding modules

The CBM *per se* typically consists of more than about 30 and less than about 250 amino acid residues. The CBM may be a "Carbohydrate-Binding Module of Family 20" or a CBM-20 module, typically a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1
10 by Joergensen et al (1997) in *Biotechnol. Lett.* 19:1027-1031, where the CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683.

The CBM (or SBM) may be derived from a starch degrading enzyme (amylolytic enzyme), such as a glucoamylase (EC 3.2.1.3), a cyclodextrin glucanotransferases or CGTase
15 (EC 2.4.1.19), an alpha-amylase (EC 3.2.1.1) or a maltogenic alpha-amylase (EC 3.2.1.133). The CBM may be derived from fungal, bacterial or plant sources, e.g. derived from *Aspergillus* sp., *Bacillus* sp., *Klebsiella* sp., or *Rhizopus* sp. The CBM may also be in the form of a non-hydrolytic polysaccharide-binding protein, e.g. found in algae, such as red alga *Porphyra purpurea*. The CBM may be located at the N or C terminus or at an internal position in a polypep-
20 tide (e.g. an enzyme).

Further examples of CBMs are described in PCT/US2004/020499.

Combination of amylases

A combination of two amylases may be added to dough to achieve anti-staling in a
25 product made from the dough.

The first amylase may be a thermostable amylase which includes a CBM, particularly a fungal amylase. It may be an alpha-amylase which retains more than 50% activity after 15 min incubation at 62°C (or 64°C or 66°C) in 50 mM sodium acetate, 1 mM CaCl₂, pH 5.7. It may have less than 50% activity at 71°C (or 69°C) under the same conditions. An example is
30 the *Valsaria* amylase described above.

The second amylase may be an exo-amylase. It may be capable of hydrolyzing starch by cleaving off linear maltooligosaccharides, e.g. maltose, maltotriose or maltotetraose, from the non-reducing ends of amylopectin. One example is maltogenic alpha-amylase (EC 3.2.1.133) such as Novamyl® or a variant thereof, e.g. having at least 90 % amino acid identity
35 to Novamyl as described in US 6162628, where the Novamyl sequence is shown as SEQ ID NO: 1.

The exo-amylase may hydrolyze amylose (e.g. wheat amylose or synthetic amylose) so that the average molecular weight of the amylose after 0.4-4 % hydrolysis (i.e. between 0.4-4 % hydrolysis of the total number of bonds) is more than 50 % (particularly more than 75 %) of the value before the hydrolysis. The hydrolysis can be conducted at the conditions described above, and the molecular weight distribution before and after the hydrolysis can be determined by HPLC. The test may be carried out as described in C. Christophersen et al., Starch 50 (1), 39-45 (1998).

Recombinant expression vector

The expression vector of the invention typically includes a selectable marker and control sequences encoding a promoter, a 5' untranslated leader and, a transcription terminator. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The polypeptide of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the amylase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism may particularly be a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum* or *S. cerevisiae*.

Hybridization

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involve presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ³²P-dCTP-labeled (specific activity > 1 x 10⁹ cpm/µg) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more particularly at least 60°C, more particularly at least 65°C, even more particularly at least 70°C, especially at least 75°C. Molecules to which the oligonucleotide probe hybridizes under these conditions may be detected using a x-ray film.

Alignment and identity

The polypeptide and polynucleotide of the invention may have identities to the disclosed sequences of at least 80 %, particularly at least 85 % or at least 90 %, e.g. at least 95 %.

5 For purposes of the present invention, alignments of sequences and calculation of identity scores may be done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in
10 a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Dough

15 The dough of the invention generally comprises flour, particularly wheat flour. The dough may be fresh, frozen or par-baked. It may be a laminated dough.

The dough may also comprise other conventional dough ingredients, e.g.: proteins, such as milk powder and gluten; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or
20 ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough may comprise fat (triglyceride) such as granulated fat or shortening.

Additional enzyme

Optionally, one or more additional enzymes may be added to the dough together with
25 the amylase(s) described above. The additional enzyme may be a lipolytic enzyme (e.g. as described in WO 9953769) or a xylanase.

Dough-based product

The invention provides a method for preparing a dough-based product by leavening the dough and heating it, e.g. by baking or steaming. The dough may be leavened e.g. by add-
30 ing chemical leavening agents or yeast, usually *Saccharomyces cerevisiae* (baker's yeast). The product may be of a soft or a crisp character, either of a white, light or dark type. Examples are steamed or baked bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls.

Amylase Units (AmU)

Amylase activity was assayed by incubating with Phadebas amylase test tablets (product of Amersham Pharmacia) suspended in 50 mM sodium acetate + 1 mM CaCl₂ at pH 5.7 and determining OD at 650 nm. The Amylase Unit (AmU) was defined by taking the activity
5 of the commercial product BAN 480L (product of Novozymes A/S) as 480 AmU.

EXAMPLES

Materials and methods

RNeasy Mini Kit (Qiagen, Cat. #74904).
Taq DNA polymerase (Promega, Cat. # M166A)
10 pGEM-T Vector System I (Promega, Cat. # A3600)
WizardTM Plus Minipreps DNA Purification System (Promega, Cat. # A7510)
5' Rapid Amplification of cDNA End System (Life Technologies, 5'RACE, Cat. # 18374-041),
3' Rapid Amplification of cDNA End System (Life Technologies , 3' RACE, Cat. #
15 1085805)
ElectroMAXTM DH10B Cells (Life Technologies, Cat. # 18290-015)

Example 1: Cultivation of fungal strain for cDNA preparation

A fungal strain of *Valsaria rubricosa* was grown on YG agar plate (4.5 cm diam) for 5 days under 37°C in the darkness and used for inoculating shake flask. The plates with fully
20 grown cultures were stored at 4°C before use.

To obtain the mycelium for cDNA library construction, 4-6 agar plugs with fully grown fungal cultures on the YG agar plates were used to inoculate one shake flask with FG-4 (50 ml in 500 ml Erlenmeyer flask with 2 baffles: 30 g Soymeal, 15 g Maltose, 5 g Peptone, 1000 ml H₂O, 1% starch, 1 g olive oil (2 drops / flask); Autoclave at 121 °C for 30 min) and grown under
25 37°C, 160 rpm for 24 hours. The mycelium was harvested by centrifugation of the culture broth at 8000 rpm and 4°C for 30 minutes. Then mycelium was transferred into a clean plastic bag following by immediately freezing in liquid nitrogen and stored at -80°C before total RNA was isolated.

Probe designing:

30 Degenerate primers were designed based on alignment of already known amylase gene sequences: amyD1 (SEQ ID NO: 3) and amyD2R (SEQ ID NO: 4).

The N-terminal amino acid sequence of the purified amylase AM835F was determined as shown in SEQ ID NO: 13. This was used to design four degenerate primers: AM835n-s1

(SEQ ID NO: 5), AM835n-s2a (SEQ ID NO: 6), AM835n-s2b (SEQ ID NO: 7), AM835n-s2c (SEQ ID NO: 8).

Extraction of total RNA:

Total RNA was isolated from the frozen mycelium of a strain of *Valsaria rubricosa* by using RNeasy Mini Kit according to the manufacturer's instructions.

Gene cloning:

cDNA was synthesized using 3' RACE kit. The primary PCR was performed by using N-terminal based degenerate primers (primer AM835n-s2 is a mixture of AM835n-s2a, b and c) with AUAP provided by the 3'RACE kit:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
100 micro-M AM835n-s2a	1 micro-l
100 micro-M AM835n-s2b	1 micro-l
100 micro-M AM835n-s2c	1 micro-l
AUAP	1 micro-l
cDNA	2 micro-l
Taq DNA polymerase (5u/ micro-l)	1 micro-l
H ₂ O	34 micro-l

10 The PCR program was: 94°C for 3 min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1.5 min; final extension at 72°C for 10 min.

There was no specific amplification seen when the PCR product was visualized under UV but this product was used for second PCR with degenerate primers designed based on amylase homology. The 2nd PCR was performed by using amylase probes (amyD1 and amyD2R) and using the primary PCR as template:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
100 micro-M amyD1	1 micro-l
100 micro-M amy D2R	1 micro-l
1 st PCR	1 micro-l
Taq DNA polymerase (5u/ micro-l)	1 micro-l
H ₂ O	37 micro-l

The PCR program was: 94°C for 3min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1min; final extension at 72°C for 10min. A specific band was amplified at ~700bps and this was confirmed to be amylase by sequencing.

Based on the above obtained partial sequence, new primers were designed for 5' and 3' end cloning. For 5' end cloning, after cDNA was synthesized initiated with amy835as1 (SEQ ID NO: 9) by 5'RACE kit, PCR was performed with primer pairs amy835as1 and AAP (provided by the kit). Then nested PCR was performed with primer pair amy835as3 (SEQ ID NO: 14) and AUAP by using primary PCR (amy835as1-AAP) as template. A fragment of ~600bp was obtained and confirmed by sequencing. For 3' end cloning, PCR was performed by using primer pair of amy835f1 (SEQ ID NO: 15) and AUAP and cDNA as template. The nested PCR was performed by using primer pair amy835f2 (SEQ ID NO: 10) with AUAP and 1st PCR as template. A fragment at ~600 bps was amplified and again confirmed by sequencing.

10 Then based on the cloned 5' and 3' end sequences, the 5' and 3' end primers for full length cloning was designed and used for full length cloning of the amylase AM835. By using cDNA synthesized by 3' RACE kit as template and probes amy835s00 (SEQ ID NO: 11) and amy835as01 (SEQ ID NO: 12) as primers:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
10 micro-M amy835s00	1 micro-l
10 micro-M amy835as01	1 micro-l
cDNA	2 micro-l
Taq DNA polymerase (5 u/micro-l)	1 micro-l
H ₂ O	36 micro-l

15 PCR program was: 94°C for 3 min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1.5 min; final extension at 72°C for 10 min.

A specific fragment of ~2.0 kb was PCR-amplified. The fragment was cloned into pGEM-T vector (Promega) which has a 3'-T overhang and transformed into *E.coli* DH10B (ElectroMAX DH10B Cells, available from Life Technologies, Cat. # 18290-015) and further sequenced.

20 Example 2: Production of amylase

YG and FG-4 media were prepared as follows:

YG: Yeast-glucose agar

5.0 g Difco powdered yeast extract; 10.0 g glucose
20.0 g agar; 1000 ml tap water
25 Autoclave at 121°C for 15-20 min.

FG-4 Media 50 ml / flask:

30 g Soymeal, 15 g Maltose

5 g Peptone, 1000 ml H₂O
 1 g olive oil (2 drops / flask)

50 ml in 500 ml Erlenmeyer flask with 2 baffles. Autoclave at 121 °C for 30 min.

A strain of the thermophilic fungus *Valsaria rubricosa* was grown on YG agar plate
 5 (4.5 cm diam) for 3 days under 37°C in the darkness and used for inoculating shake flask. The
 plates with fully grown cultures were stored at 4°C before use.

For enzyme production, 4-6 agar plugs with fully grown fungal cultures on the above
 plates were used to inoculate one shake flask with FG-4 and grown under 37°C, 160 rpm for
 72 hours, then harvested by centrifuged the culture broth at 8000 rpm and 4°C for 30 minutes.
 10 The supernatant was collected and used for enzyme purification.

1000 ml supernatant was precipitated with ammonium sulfate (80% saturation) and
 redissolved in 100 ml 25mM Tris-HCl buffer, pH7.0, then dialyzed against the same buffer and
 filtered through a 0.45 mm filter, the final volume was 200 ml. The solution was applied to a 35
 ml Source 15Q column (Pharmacia) equilibrated in 25 mM Tris-HCl buffer, pH7.0, and the pro-
 15 teins was eluted with a linear NaCl gradient (0 –0.3M). Fractions from the column were ana-
 lyzed for amylase activity on AZCL-amylose at pH 5.5. Fractions with amylase activity were
 pooled. Then the pooled solution was ultrafiltrated, the concentrated solution was applied to a
 180ml SuperdexTM75 column equilibrated with 25 mM Tris-HCl, pH7.0, the proteins was eluted
 with the same buffer. Amylase containing fractions were analyzed by SDS-PAGE and pure
 20 fractions were pooled.

The purified amylase was used for characterization in the following example.

Example 3: Expression of an amylase from *Valsaria rubricosa* in *Aspergillus oryzae*

The DNA sequence of the *Valsaria rubricosa* amylase (SEQ ID NO.: 1) was used to
 design primers for PCR amplification of the amylase encoding-gene from the clone described
 25 in Example 1, with appropriate restriction sites added to the primer ends to facilitate sub-
 cloning of the PCR product (primers AM835.1 and AM835.2, SEQ ID NO: 16 and 17). PCR
 amplification was performed using AmpliTaqTM Gold DNA Polymerase (Applied Biosystems, Fos-
 ter City, CA, USA) following the manufacturer's instructions and using an annealing tempera-
 ture of 55°C for the first 5 cycles and 65°C for an additional 25 cycles and an extension time of
 30 2 minutes.

The PCR fragment was restricted with *Bam*HI and *Xho*I and cloned into the *Aspergill-*
lus expression vector pMStr57 using standard techniques. The expression vector pMStr57
 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to
 the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has
 35 sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergil-*
lus. Specifically, selection in *Aspergillus* is facilitated by the *amdS* gene of *Aspergillus nidu-*

lans, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (*tpi*) encoding-gene from *Aspergillus nidulans*, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The amylase-encoding gene of the resulting *Aspergillus* expression construct, pMStr91, was sequenced and the sequence agreed completely with that determined previously.

The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr91 using standard techniques (Christensen, T. et al., (1988), Biotechnology 6, 1419-1422). Transformants were cultured in YP+2%G medium shaken at 250 RPM at 30°C and expression of amylase was monitored by SDS-PAGE.

Medium YP+2%G

10g yeast extract
20g peptone
15 water to 1L
autoclave at 121°C, 20 minutes
add 100ml 20% sterile glucose solution

Example 4: Characterization of amylase

The molecular weight of the amylase prepared in a previous example was found to be around 66 kDa as seen on SDS-PAGE. The isoelectric point (pI) was found to be around pH 3.5, as determined by isoelectric focusing (IEF).

pH and temperature profiles were determined with AZCL-amylose (product of Megazyme) as substrate. At 50°C, the amylase was found to be active at pH 4-10 with an optimum around pH 5-7. At pH 5.5, the amylase was found to be active at 20-70°C with an optimum around 60°C. Thus, the *Valsaria rubricosa* amylase has a wider pH range and a higher temperature optimum than the fungal amylase from *Aspergillus oryzae*.

Stability of the amylase was determined by incubation at pH 5-7 and 60-80°C for 5-25 minutes. The results showed more than 90 % residual activity after 20 minutes at pH 6-7 and 60°C. At pH 5.0 and 60°C, the amylase was nearly completely inactivated in 15 minutes. At 70°C, the amylase was nearly completely inactivated at in 5-10 minutes at pH 6-7. It was found that at all conditions the *Valsaria rubricosa* amylase is more stable than the fungal amylase from *Aspergillus oryzae*.

The amylase showed no activity on the following substrates at pH 7.0: AZCL-galactomannan, AZCL-beta-glucan, AZCL-dextran, AZCL-xyloglucan, AZCL-potato galactan, AZCL-arabinan, AZCL-pullulan, AZCL-xylan, AZCL-he-cellulose and AZCL-casein.

Example 5: Effect of amylase on freshness of bread

Bread were baked according to the sponge & dough method.

RecipesSponge % on flour basis

Soya oil	2,5
Sodium stearoyl lactylate (SSL)	0,38
Yeast	5
Wheat flour	60
Water	62

Dough % on flour basis

Ascorbic acid	optimized for each flour
ADA	20 ppm
Salt	2
Syrup	7 (dry substance)
Water	optimized for each flour
Wheat flour	40
Calcium propionate	0.25
Enzymes	as indicated below

Sponge

- 5 Scaling of ingredients, addition of yeast, water, flour, SSL and oil into mixer bowl
 Mixing 90 rpm for 1 minutes, 150 rpm for 4 minutes
 The sponge was weighted, the temperature was measured and the sponge was placed in a bowl ~ fermentation 3 hours at 27 C, 86 % RH

Dough

- 10 Addition of ingredients and the sponge into the mixer bowl. The sponge and ingredients were mixed together 90 rpm for 9 minutes
 The temperature was measured, dough characteristics were evaluated, the dough was scaled into smaller pieces of 435 g each.
 The dough rests on the table for 10 minutes
 Doughs were sheeted and molded.
 Fermentation for 55 minutes at 42°C and 86% RH.
 Bread were baked at 200°C for 22 minutes
 Enzymes were dosed at 400 MANU/kg of Novamyl together with 0, 5 or 20 AmU/kg of the amylase of SEQ ID NO: 2 (prepared as in Example 1).

Bread were stored at room temperature until analysis.

Texture and water migration by NMR were measured on day 7, 14 and 21. A small sensory evaluation of softness and moistness was performed on day 21.

Results

- 5 Firmness of the loaves was measured as described in WO 9953769 The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Firmness after 7 days g	Firmness after 14 days g	Firmness after 21 days g
400	0	593	869	1103
400	5	505	814	1000
400	20	480	789	939

Elasticity of the loaves was measured as described in US 6162628. The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Elasticity after 7 days %	Elasticity after 14 days %	Elasticity after 21 days %
400	0	50.7	46.5	45.2
400	5	50.1	46.7	44.7
400	20	50.7	47.2	46.0

10

The data show that the amylase of the invention has a significant effect on firmness in combination with Novamyl, furthermore the elasticity seems to be comparable to or even better than that of Novamyl after 21 days of storage.

- 15 The mobility of free water was determined as described by P. L. Chen, Z. Long, R. Ruan and T. P. Labuza, Nuclear Magnetic Resonance Studies of water Mobility in Bread during Storage. Lebensmittel Wissenschaft und Technologie 30, 178-183 (1997). The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Free water after 7 days Micro-sec	Free water after 14 days Micro-sec	Free water after 21 days Micro-sec
400	0	7498	6921	6198
400	5	7780	6856	6424
400	20	7945	7004	6618

The data show that the amylase of the invention increases the amount of free water. The amount of free water has been described in literature to correlate to moistness of bread crumb.

The ranking from the small sensory evaluation of softness and moistness on day 21
5 showed the following ranking (MANU/kg of Novamyl + AmU/kg of amylase of invention):

Moistest: 400 MANU + 20 AmU

Second: 400 MANU + 5 AmU

Lowest (least moist): 400 MANU

14a

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14c

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14e

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14f

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14m

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Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Asp Asn His Asp Asn Pro	
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Trp Arg Ser Gly Tyr Ser Thr Thr Gly Asp Ile Tyr Thr Thr Ile Ala	
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gcc ctc tcc gcc gcg cgc acc gcg gcc ggt ggc ctc gcc ggt aac gat	1155
Ala Leu Ser Ala Ala Arg Thr Ala Ala Gly Gly Leu Ala Gly Asn Asp	
345 350 355 360	

14n

cac gtt cac ctg tac aca acc gac aac gcg tac gcc tgg tcc cgt gcg His Val His Leu Tyr Thr Thr Asp Asn Ala Tyr Ala Trp Ser Arg Ala 365 370 375	1203
agc ggc aag ctc atc gtc gtc acg tcc aac cgc ggc agc tcc gac agc Ser Gly Lys Leu Ile Val Val Thr Ser Asn Arg Gly Ser Ser Asp Ser 380 385 390	1251
agc acc atc tgc ttc agc acc cag cag gcc agc ggc acc acc tgg acc Ser Thr Ile Cys Phe Ser Thr Gln Gln Ala Ser Gly Thr Thr Trp Thr 395 400 405	1299
agc acg atc acc ggc aac tcg tac acc gcc gac agc aac ggc cag atc Ser Thr Ile Thr Gly Asn Ser Tyr Thr Ala Asp Ser Asn Gly Gln Ile 410 415 420	1347
tgc gtg cag ctg tcc agc ggc ggg ccc gag gcg ctc gtc gtc tcc acc Cys Val Gln Leu Ser Ser Gly Gly Pro Glu Ala Leu Val Val Ser Thr 425 430 435 440	1395
gcg acc ggc acg gcc acg gcg acg acc ctg tcc acg acc acc aag acg Ala Thr Gly Thr Ala Thr Ala Thr Thr Leu Ser Thr Thr Thr Lys Thr 445 450 455	1443
tcc acc tcg acc gcc tcc tgc gcc gcc acc gtc gcc gtc acc ttc aat Ser Thr Ser Thr Ala Ser Cys Ala Ala Thr Val Ala Val Thr Phe Asn 460 465 470	1491
gag ctc gtc acc acc aac tac ggc gac acc atc cgc ctg acg ggc tcc Glu Leu Val Thr Thr Asn Tyr Gly Asp Thr Ile Arg Leu Thr Gly Ser 475 480 485	1539
atc tcc cag ctc agc agc tgg agc gca acc tcc ggg ctg gcc ctg agc Ile Ser Gln Leu Ser Ser Trp Ser Ala Thr Ser Gly Leu Ala Leu Ser 490 495 500	1587
gcg tcc gcg tac acg tcc agc aac ccg ctc tgg agc gtg acg gtc agc Ala Ser Ala Tyr Thr Ser Ser Asn Pro Leu Trp Ser Val Thr Val Ser 505 510 515 520	1635
ctg cct gcc ggc acg tcg ttc gag tac aag ttc gtc cgc atc acg agc Leu Pro Ala Gly Thr Ser Phe Glu Tyr Lys Phe Val Arg Ile Thr Ser 525 530 535	1683
gac ggc acc gtc acc tgg gaa tcg gac ccg aac cgc agc tac acc gtc Asp Gly Thr Val Thr Trp Glu Ser Asp Pro Asn Arg Ser Tyr Thr Val 540 545 550	1731
ccg acg tgc gcg agc acc gca acg atc agc aat acc tgg cgg Pro Thr Cys Ala Ser Thr Ala Thr Ile Ser Asn Thr Trp Arg 555 560 565	1773
tgagctgtgg atgtatctgt atctgtacat atgagaggtc tttgagaggc cagggcgggt	1833
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tggtg	1898

14o

<210> 19
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 <212> PRT
 <213> Valsaria rubricosa

<400> 19
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 Gln Leu Leu Ala Ala Ser Asn Ser Asp Trp Arg Ser Arg Asn Ile Tyr
 -1 1 5 10
 Phe Ala Leu Thr Asp Arg Val Ala Asn Pro Ser Thr Thr Thr Ala Cys
 15 20 25
 Ser Asp Leu Ser Asn Tyr Cys Gly Gly Thr Trp Ser Gly Leu Ser Ser
 30 35 40
 Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Asp Ser Ile Trp Ile Thr
 45 50 55 60
 Pro Val Val Glu Asn Cys Asp Gly Gly Tyr His Gly Tyr Trp Ala Lys
 65 70 75
 Ala Leu Tyr Asn Val Asn Thr Asn Tyr Gly Ser Ala Asp Asp Leu Lys
 80 85 90
 Asn Phe Val Ala Ala Ala His Ala Lys Gly Met Tyr Val Met Val Asp
 95 100 105
 Val Val Ala Asn His Met Gly Ser Cys Gly Ile Ala Asn Leu Ser Pro
 110 115 120
 Pro Pro Leu Asn Glu Glu Ser Ser Tyr His Thr Gln Cys Asp Ile Asp
 125 130 135 140
 Tyr Ser Ser Gln Ser Ser Ile Glu Thr Cys Trp Ile Ser Gly Leu Pro
 145 150 155
 Asp Leu Asp Thr Thr Asp Ser Thr Ile Arg Ser Leu Phe Gln Thr Trp
 160 165 170
 Val His Gly Leu Val Ser Asn Tyr Ser Phe Asp Gly Leu Arg Val Asp
 175 180 185
 Thr Val Lys His Val Glu Lys Asp Tyr Trp Pro Gly Phe Val Ser Ala
 190 195 200
 Ala Gly Thr Tyr Ala Ile Gly Glu Val Phe Ser Gly Asp Thr Ser Tyr
 205 210 215 220
 Val Ala Gly Tyr Gln Ser Val Met Pro Gly Leu Leu Asn Tyr Pro Ile
 225 230 235
 Tyr Tyr Pro Leu Ile Arg Val Phe Ala Gln Gly Ala Ser Phe Thr Asp
 240 245 250

14p

Leu	Val	Ser	Asn	His	Asp	Ser	Val	Gly	Ser	Thr	Phe	Ser	Asp	Pro	Thr
		255					260					265			
Leu	Leu	Gly	Asn	Phe	Ile	Asp	Asn	His	Asp	Asn	Pro	Arg	Phe	Leu	Ser
	270					275					280				
Tyr	Thr	Ser	Asp	His	Ala	Leu	Leu	Lys	Asn	Ala	Leu	Ala	Tyr	Val	Ile
285					290					295					300
Leu	Ala	Arg	Gly	Ile	Pro	Ile	Val	Tyr	Tyr	Gly	Thr	Glu	Gln	Gly	Tyr
				305					310					315	
Ser	Gly	Ser	Ser	Asp	Pro	Ala	Asn	Arg	Glu	Asp	Leu	Trp	Arg	Ser	Gly
			320					325					330		
Tyr	Ser	Thr	Thr	Gly	Asp	Ile	Tyr	Thr	Thr	Ile	Ala	Ala	Leu	Ser	Ala
		335					340					345			
Ala	Arg	Thr	Ala	Ala	Gly	Gly	Leu	Ala	Gly	Asn	Asp	His	Val	His	Leu
	350					355					360				
Tyr	Thr	Thr	Asp	Asn	Ala	Tyr	Ala	Trp	Ser	Arg	Ala	Ser	Gly	Lys	Leu
365					370					375					380
Ile	Val	Val	Thr	Ser	Asn	Arg	Gly	Ser	Ser	Asp	Ser	Ser	Thr	Ile	Cys
				385					390					395	
Phe	Ser	Thr	Gln	Gln	Ala	Ser	Gly	Thr	Thr	Trp	Thr	Ser	Thr	Ile	Thr
			400					405					410		
Gly	Asn	Ser	Tyr	Thr	Ala	Asp	Ser	Asn	Gly	Gln	Ile	Cys	Val	Gln	Leu
		415					420					425			
Ser	Ser	Gly	Gly	Pro	Glu	Ala	Leu	Val	Val	Ser	Thr	Ala	Thr	Gly	Thr
	430					435					440				
Ala	Thr	Ala	Thr	Thr	Leu	Ser	Thr	Thr	Thr	Lys	Thr	Ser	Thr	Ser	Thr
445					450					455					460
Ala	Ser	Cys	Ala	Ala	Thr	Val	Ala	Val	Thr	Phe	Asn	Glu	Leu	Val	Thr
				465					470					475	
Thr	Asn	Tyr	Gly	Asp	Thr	Ile	Arg	Leu	Thr	Gly	Ser	Ile	Ser	Gln	Leu
			480					485					490		
Ser	Ser	Trp	Ser	Ala	Thr	Ser	Gly	Leu	Ala	Leu	Ser	Ala	Ser	Ala	Tyr
		495					500					505			
Thr	Ser	Ser	Asn	Pro	Leu	Trp	Ser	Val	Thr	Val	Ser	Leu	Pro	Ala	Gly
	510					515					520				
Thr	Ser	Phe	Glu	Tyr	Lys	Phe	Val	Arg	Ile	Thr	Ser	Asp	Gly	Thr	Val
525					530					535					540
Thr	Trp	Glu	Ser	Asp	Pro	Asn	Arg	Ser	Tyr	Thr	Val	Pro	Thr	Cys	Ala
				545					550					555	

14q

Ser	Thr	Ala	Thr	Ile	Ser	Asn	Thr	Trp	Arg
			560					565	

CLAIMS:

1. A polypeptide which has amylase activity and has an amino acid sequence which comprises:

- a) a sequence as shown in positions 1-439 of SEQ ID NO:2 or 19; or
- b) a sequence which has at least 70 % identity to the sequence as shown in positions 1-439 of SEQ ID NO:2 or at least 80% identity to the sequence as shown in positions 1-439 of SEQ ID NO:19.

2. The polypeptide of claim 1, wherein the amino acid sequence further comprises a carbohydrate-binding domain.

3. The polypeptide of claim 1 or 2, which has an amino acid sequence comprising:

- a) a sequence as shown in positions 1-566 of SEQ ID NO:2 or 19; or
- b) a sequence which has at least 70 % identity to the sequence as shown in positions 1-566 of SEQ ID NO:2 or at least 80 % identity to the sequence as shown in positions 1-566 of SEQ ID NO:19.

4. A polypeptide which has an amino acid sequence comprising a carbohydrate-binding module having at least 70 % identity to amino acids 440-566 of SEQ ID NO:2 or 19.

5. A polynucleotide comprising a sequence which encodes the polypeptide of any one of claims 1-4.

6. A polynucleotide encoding for an amylase comprising a sequence which is a sequence which has at least 70 % identity to nucleotides 146-1462 of SEQ ID NO:1 or at least 80 % identity to nucleotides 76-1392 of SEQ ID NO:18 and encodes an amylase catalytic core.

7. A polynucleotide encoding for an amylase comprising a sequence which is a sequence which has at least 70 % identity to nucleotides 146-1843 of SEQ

ID NO:1 or nucleotides 76-1773 of SEQ ID NO:18 and encodes an amylase catalytic core and a carbohydrate binding domain.

8. A vector comprising the polynucleotide of any one of claims 5-7 operably linked to one or more control sequences that direct the production of the polypeptide of any one of claims 1-4 or the amylase of claim 6 or 7 in a suitable host.

9. A host cell transformed with the vector of claim 8.

10. A method for producing an amylase, which comprises:

a) cultivating the host cell of claim 9 under conditions appropriate for expression of amylase, and

b) recovering the amylase.

11. A dough composition which comprises flour and the polypeptide of any one of claims 1-4.

12. A process for preparing a dough-based product, comprising adding the polypeptide of any one of claims 1-4 to a dough, leavening, and heating the dough.

13. The process of claim 12, further comprising adding an exo-acting amylase to the dough.

14. The process of claim 13, wherein the exo-acting amylase is a maltogenic alpha-amylase.